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Infection With an Unenveloped DNA Virus (TTV) Associated With Posttransfusion Non-A to G Hepatitis in Hepatitis Patients and Healthy Blood Donors in Thailand

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An unenveloped single-stranded DNA virus (TTV) has been reported in association with posttransfusion and acute and chronic hepatitis of unknown etiology. DNA of TTV was tested for by polymerase chain reaction with heminested primers in 127 patients with chronic liver disease and 105 healthy blood donors in Thailand. TTV DNA was detected in 23 (59%) of the 39 patients without hepatitis B surface antigen or RNA of hepatitis C virus, at a frequency significantly higher than the detection in 21 (36%) of the 59 patients with HBsAg ($P < 0.05$) or in 38 (36%) of the 105 blood donors ($P < 0.05$). Among patients with chronic liver disease, TTV DNA occurred in those with liver cirrhosis and hepatocellular carcinoma more frequently than in those with chronic hepatitis (35 of 65 or 54% vs. 20 of 62 or 32%, $P < 0.05$). There were no differences in age, sex, or markers of infection with hepatitis B, C and GBV-C/HGV viruses, indicating a mode of transmission of TTV different from those of the other hepatitis viruses. Phylogenetic analysis indicated three different genotypes of TTV with six distinct subtypes in Thailand. Based on these results, TTV would have a role in the development of chronic liver disease of unknown etiology in Thailand. ***J. Med. Virol. 56:234–238, 1998.***

KEY WORDS: hepatitis viruses; genotypes; chronic hepatitis; liver cirrhosis; hepatocellular carcinoma

INTRODUCTION

The past 30 years have witnessed significant advances in research on viral hepatitis [Purcell, 1993]. Five hepatitis viruses were identified: hepatitis A virus, hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus, and hepatitis E virus.

Since the etiology of hepatitis can be diagnosed by detecting specific serum markers of the five hepatitis viruses, an entity of hepatic disease is being diagnosed that is not attributable to any of the recognizable hepatitis viruses. Such a category of liver disease is labeled non-A to E, and a viral origin is strongly suspected for it. As a candidate of a putative non-A to E hepatitis virus, GB virus C [Simons et al., 1995] and hepatitis G virus (HGV) [Linnen et al., 1996] have been proposed. GBV-C and HGV are separate isolates of the same virus [Zuckerman, 1995] and are referred to collectively as GBV-C/HGV.

Clinical and epidemiological studies, however, have not been able to ascribe any substantial role to GBV-C/HGV in inducing hepatitis [Alter, 1997; Miyakawa and Mayumi, 1997]. There is no proof to support replication of GBV-C/HGV in the liver [Laskus et al., 1997]. Hence, there may well be unidentified viral agents that induce hepatitis.

We have discovered a DNA virus in the circulation of patients with non-A to G hepatitis in close association with elevated levels of serum alanine aminotransferase (ALT) [Nishizawa et al., 1997]. This virus was named TT virus (TTV) after the initials of a patient from whom the original clone was recovered. TTV is an unenveloped, single-stranded DNA virus and detected significantly more frequently in patients with acute or chronic hepatitis of unknown etiology than in blood do-

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nors (46% vs. 12%) in Japan [Okamoto et al., 1998a]. TTV may replicate in liver because its DNA is detected at titers 10- to 100-fold higher than in the corresponding serum from patients with chronic non-A to G hepatitis.

In order to assess the epidemiology and the role of TTV in hepatitis patients in Asia, sera from chronic liver disease and healthy blood donors in Thailand were examined for TTV DNA by polymerase chain reaction (PCR) with heminested primers. In addition, TTV DNA clones obtained were analyzed phylogenetically to establish the viral genotypes prevalent in Thailand.

MATERIALS AND METHODS

Patients

A total of 127 patients with chronic liver disease in Thailand with a mean ± S.D. age of 46 ± 13, including 89 males and 38 females, were studied. They have been included in a study for markers of HCV infection in a previous study [Luengrojanakul et al., 1994], and comprised 62 patients with chronic hepatitis (CH), 50 with liver cirrhosis (LC), and 15 with hepatocellular carcinoma (HCC). Blood donors in the districts where the patients lived served as controls. They were aged 41 ± 14 years and included 100 males and 5 females.

Serological Markers of Hepatitis Viruses

Hepatitis B surface antigen (HBsAg) and the corresponding antibody (anti-HBs) were determined by passive hemagglutination (MyCell: Institute of Immunology Co., Tokyo, Japan). Antibody to hepatitis B core (anti-HBc) was determined by hemagglutination inhibition by the method described previously [Iizuka et al., 1992]. RNA of HCV [Okamoto et al., 1994] and RNA of GBV-C/HGV [Shimizu et al., 1996] were determined by reverse transcription-PCR with nested primers deduced from well-conserved segments in the 5′ untranslated region.

Determination of TTV DNA

Serum (50 μl) was pretreated with proteinase K and sodium dodecyl sulfate, and nucleic acids were extracted with phenol and chloroform as described previously [Okamoto et al., 1990]. Extracted nucleic acids were dissolved in 20 μl of Tris-HCl buffer (10 mM, pH 8.0) supplemented with 1 mM EDTA, heated at 95°C for 15 min and quickly chilled on ice. A half amount was tested for TTV DNA by PCR with heminested primers, which are compatible with different genotypes and subtypes [Okamoto et al., 1998a], and Perkin-Elmer AmpliTaq DNA Polymerase (Roche Molecular Systems, New Jersey) by the method described elsewhere [Okamoto et al., 1998b].

In essence, the first round of PCR was carried out with NG059 primer (sense: 5′-ACAGACAGAGGAGAAGGCAACATG-3′) and NG063 (antisense: 5′-CTGGCATTTTACCATTTCCAAAGTT-3′) for 35 cycles (94°C for 30 sec; 60°C for 45 sec; 72°C for 45 sec, with additional 7 min for the last cycle), and the second-round PCR with NG061 (sense: 5′-GGCAACATGYTRTGGATAGACTGG-3′, Y = T or C; R = A or G) and NG063 for 25 cycles with the same conditions. The amplification products by the first-round PCR measured 286 base pairs (bp), and those by the second-round 271 bp.

Determination of a Partial TTV DNA Sequence

Products of the second-round PCR were ligated to pT7BlueT-Vector (Novagen Inc., Wisconsin), and, using plasmid DNA extracted from transformed *E. coli,* both strands were sequenced with ThermoSequenase fluorescent-labeled primer cycle sequencing kit (Amersham International, Buckinghamshire, U.K.). The sequence was determined on three clones each for respective PCR products, and the consensus sequence was adopted.

Computer Analysis of Nucleotide Sequences

Sequence analysis of TTV isolates was undertaken using ODEN program version 1.1.1 (National Institute of Genetics, Mishima, Japan) and Genetyx-Mac version 8.0 (Software Development Co., Tokyo, Japan). A phylogenetic tree was constructed by the unweighted pair-group method with an arithmetic mean [Nei, 1987].

Statistical Analysis

The frequency between groups was compared using Fisher's exact test or chi-square test. Group means were compared using Student's *t*-test. A difference with a P value of <0.05 was considered significant.

RESULTS

TTV DNA in Patients With Chronic Liver Disease of Distinct Etiology

The prevalence of TTV DNA in patients with chronic liver disease stratified into four groups with different etiologies is indicated in Table I. TTV DNA was detected in 23 (59%) of the 39 patients without HBsAg or HCV RNA and considered cryptogenic (group A), more frequently ($P < 0.05$) than in 32 (36%) of the 88 patients with HBsAg or HCV RNA, or both (groups B, C, and D combined). The prevalence in patients without HBsAg or HCV RNA (23 of 39 or 59%) was significantly higher than in patients with HBsAg (21 of 59 or 36%, $P < 0.05$) or in healthy blood donors (38 of 105 or 36%, $P < 0.05$).

Of the 39 patients without HBsAg or HCV RNA, TTV DNA tended to be more frequent in those with LC (68%) and HCC (67%) than in those with CH (43%). Of the 39 patients, 15 were without any other serological markers of known hepatitis virus infection, such as anti-HBs, anti-HBc, anti-HCV, and GBV-C/HGV RNA. TTV DNA was detected in seven (47%), leaving only eight patients (representing 6% of the 127 studied) who were without any viral markers.

TTV DNA in Patients With Chronic Hepatitis, Cirrhosis, or Hepatocellular Carcinoma

TTV DNA was detected in 55 (43%) of the 127 patients studied. The prevalence tended to increase in

TABLE I. TTV DNA in Patients With Chronic Liver Disease and Healthy Individuals in Thailand[a]

Markers of HBV and HCV infections	Total	Age (years) mean ± S.D.	Male	TTV DNA Number (% of total)	CH	LC	HCC
Patients	127	46 ± 13	70%	55 (43)	20 of 62 (32%)	26 of 50 (52%)	9 of 15 (60%)
(A) HBsAg (–) HCV RNA (–)	39	50 ± 14	69%	23 (59)	6 of 14 (43%)	15 of 22 (68%)	2 of 3 (67%)
(B) HBsAg (+) HCV RNA (–)	59	43 ± 11	76%	21 (36)	8 of 28 (29%)	7 of 21 (33%)	6 of 10 (60%)
(C) HBsAg (–) HCV RNA (+)	28	49 ± 11	57%	10 (36)	5 of 19 (26%)	4 of 7 (57%)	1 of 2 (50%)
(D) HBsAg (+) HCV RNA (+)	1	39	100%	1 (100)	1 of 1 (100%)	0 of 0	0 of 0
Healthy individuals	105	41 ± 14	95%	38 (36)	NA	NA	NA

[a]Abbreviations are: CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; NA, not applicable. Number of patients among those with each disease category and percentage are given.

patients with more advanced liver disease (Table I). Thus, TTV DNA was detected in 32% of the patients with CH, 52% in those with LC, and 60% in those with HCC. The prevalence was significantly different between patients with CH and LC ($P < 0.05$) and between those with CH and HCC ($P < 0.05$). As for the other viral markers, HCV RNA was significantly more frequent ($P < 0.05$) in patients with CH (20 of 62 or 32%) than in those with either LC or HCC (9 of 65 or 14%). No differences were noted in the prevalence of the other viral markers among patients with various diseases.

Comparison of Patients With and Without TTV DNA

Table II compares various features between the 55 patients with TTV DNA and the 72 patients without it. There were no differences in demographic factors or prevalence rates of the other viral markers. On comparing various forms of chronic liver disease, CH was more frequent in patients without TTV DNA than in those with it ($P < 0.05$).

Phylogenetic Analysis of TTV in Thailand

From the 23 patients with TTV DNA but without HBsAg or HCV RNA, nine were randomly selected (cases 1–9). A TTV DNA sequence of 222 bases was determined for them and compared in Figure 1. Three patients (cases 1, 2, and 6) possessed two kinds of TTV with different sequences.

In Figure 2, a phylogenetic tree was constructed for the 12 TTV DNA sequences of the nine cases, along with the four clones of distinct genotypes/subtypes previously reported [Okamoto et al., 1998a]. A genotype other than genotypes 1 and 2 was identified, which was separated by an evolutionary distance >0.30. In addition, two new subtypes of genotype 2 were found separated from subtypes 2a and 2b by an evolutionary distance >0.15. They were not given a designation at present to avoid confusion of the nomenclature of genotypes/subtypes, which is to be expected as new sequences are reported from other areas of the world still to be studied.

TABLE II. Comparison of Patients With and Without TTV DNA

Features	TTV DNA Positive (n = 55)	Negative (n = 72)	Differences
Age (years)	46 ± 14	47 ± 13	NS
Male	39 (71%)	50 (69%)	NS
Disease[a]			
CH	20 (36%)	42 (58%)	$P < 0.05$
LC	26 (47%)	24 (33%)	NS
HCC	9 (16%)	6 (8%)	NS
Viral markers			
HBsAg	22 (40%)	38 (53%)	NS
Anti-HBV[b]	43 (78%)	56 (78%)	NS
Anti-HCV	11 (20%)	22 (31%)	NS
HCV RNA	11 (20%)	18 (25%)	NS
HGV RNA	12 (22%)	13 (18%)	NS

[a]Abbreviations are: CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; NS, not significant.
[b]Anti-HBs or anti-HBc, or both.

Three patients with two different TTV DNA sequences were found to be infected with TTV of two different genotypes. Two (cases 1 and 2) were infected with TTV of genotypes 1 and 2, and the remaining patient (case 6) with genotype 2 and a new genotype.

DISCUSSION

TTV DNA was detected at a high prevalence in apparently healthy blood donors in Thailand (38 of 105 or 36%). The prevalence was even higher in patients with chronic liver disease ranging from CH to HCC (55 of 127 or 43%). Of particular note is the detection of TTV DNA in 23 (59%) of the 39 patients who were without HBsAg or HCV RNA, indicating that TTV may have caused their liver disease. Moreover, 15 of the 39 patients were without any serological markers of known hepatitis viruses, such as anti-HBs, anti-HBc, anti-HCV, and GBV-C/HGV RNA. TTV DNA was detected in 7 (47%) of the patients, leaving only eight patients who were without any viral markers, corresponding to 6% of the 127 studied. Hence, the contribution of any unidentified hepatitis viruses, in the development of chronic liver disease in Thailand, would be low.

There were no differences in demographic factors or

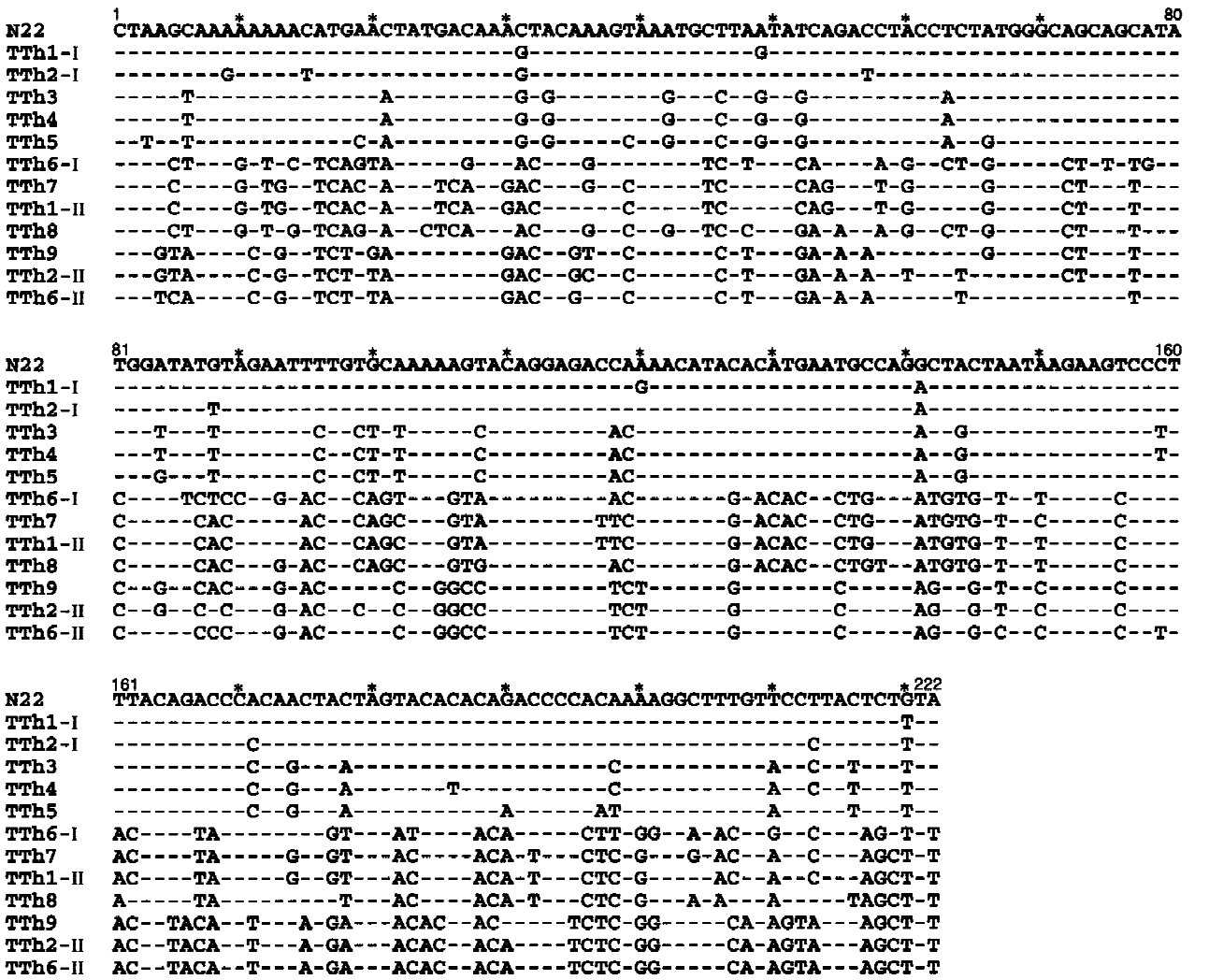

Fig. 1. Sequences of TTV DNA clones from nine hepatitis patients without HBsAg or HCV RNA in Thailand. A sequence of 222 bp in the products of the second-round PCR (primer sequences at both ends excluded) is shown. The consensus sequences of three clones from each sample are shown. The sequence of the original N22 clone [Nishizawa et al., 1997] is indicated at the top. Dashes represent nucleotides identical to those in the N22 clone. Two different sequences in cases 1, 2, and 6 are distinguished by numbers after a dash.

the prevalence of markers of the other viruses between patients with and without TTV DNA. However, TTV DNA was detected in 32 (36%) of the 88 patients with HBsAg or HCV RNA, or both, a little less frequently than in 23 (59%) of the 39 with neither markers. There is evidence for potential hepatotropism of TTV; TTV DNA is detected at titers 10- to 100-fold higher in the liver tissue than in the serum of carriers [Okamoto et al., 1998a]. Whether TTV might interfere with the replication of HBV or HCV in the liver would be a subject of future investigation.

Detection of TTV DNA increased pari passu with the severity of liver disease in patients without HBsAg or HCV RNA, as well as in patients with either HBsAg or HCV RNA. The prevalence of TTV DNA may increase with age, which needs to be taken into account in estimating such a trend. However, there were no differences in the age between patients with and without TTV DNA (46.3 ± 13.7 vs. 46.6 ± 12.6 years), nor in the blood donors with and without it (41.5 ± 16.9 vs. 41.0 ± 13.3 years). Despite this, the possibility of prolonged TTV infection leading to severe disease, as is proven for HBV and HCV, has to be evaluated in future studies.

There were no differences in demography or in the frequency of the other hepatitis virus markers (Table II). Hence, TTV may infect individuals living in Thailand by routes different from HBV or HCV. We reported that TTV DNA is secreted into feces from infected individuals [Okamoto et al., 1998b]. Although the infectivity of TTV in feces needs to be verified in animal transmission studies, this observation suggests fecal-oral transmission of TTV. There remains no doubt about a parenteral transmission of TTV, since the infection occurs in recipients of transfusions [Nishizawa et al., 1997] and is more frequent in patients with hemophilia (68%), those on maintenance hemodialysis (46%), and intravenous drug users (40%) than in blood donors (12%) in Japan [Okamoto et al., 1998a]. Non-

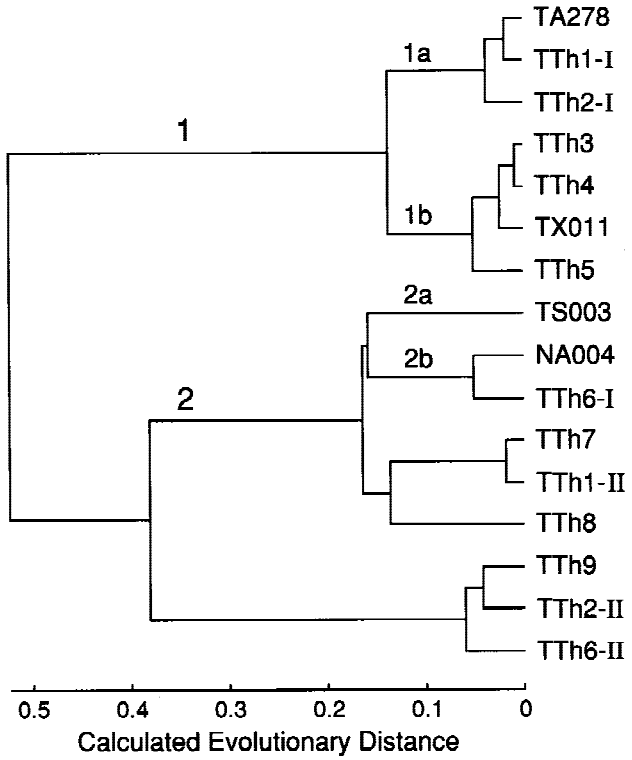

Fig. 2. A phylogenetic tree of TTV. The tree was constructed by comparison of a 222 bp sequence in TTV isolates, including one clone each of genotypes 1a, 1b, 2a, or 2b [Okamoto et al., 1998a], as well as isolates from nine cases from Thailand. Two different sequences in cases 1, 2, and 6 are distinguished by numbers after a dash.

parenteral infection of TTV, in addition to parenteral infection, would be responsible for the high prevalence of TTV DNA observed in both patients and blood donors in Thailand. In fact, the prevalence of TTV DNA in blood donors (33%) far exceeds that of HBsAg (6.3%) and HCV RNA (0.8%) reported in Thailand [Luengrojanakul et al., 1994].

TTV has a wide range of sequence divergence, which occurs in two tiers, as with HCV [Okamoto et al., 1998a, 1998b]. Divergence in a higher order is classified as genotypes and labeled with Arabic numbers, while that in a lower order as subtypes and with small-case letters. To date, two genotypes with an evolutionary distance >0.30 and five subtypes with a distance >0.15 have been recognized. Subtypes previously reported, such as 1a, 1b, and 2b, were identified in TTV isolates in Thailand. In addition, one new genotype and two additional subtypes in genotype 2 were detected in TTV isolates in Thailand. Because of the prospect of many more genotypes/subtypes prevailing in unexamined areas of the world, and also as a result of the confusion experienced with the classification of HCV [Tokita et al., 1996], the new genotype and subtypes in Thailand have not been given a designation. Whether new genotypes/subtypes of TTV are intrinsic in Thailand and associated with a particular form of liver disease would be worthy of future studies.

Two different genotypes or distinct subtypes of the same genotype were found in TTV infecting the same patients in three of the nine cases studied. They would have occurred either by infection with inocula harboring two different kinds of TTV or by superinfection introduced into the patients who were infected already. This will be established by following up carriers of TTV for the development of new genotypes and subtypes.

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